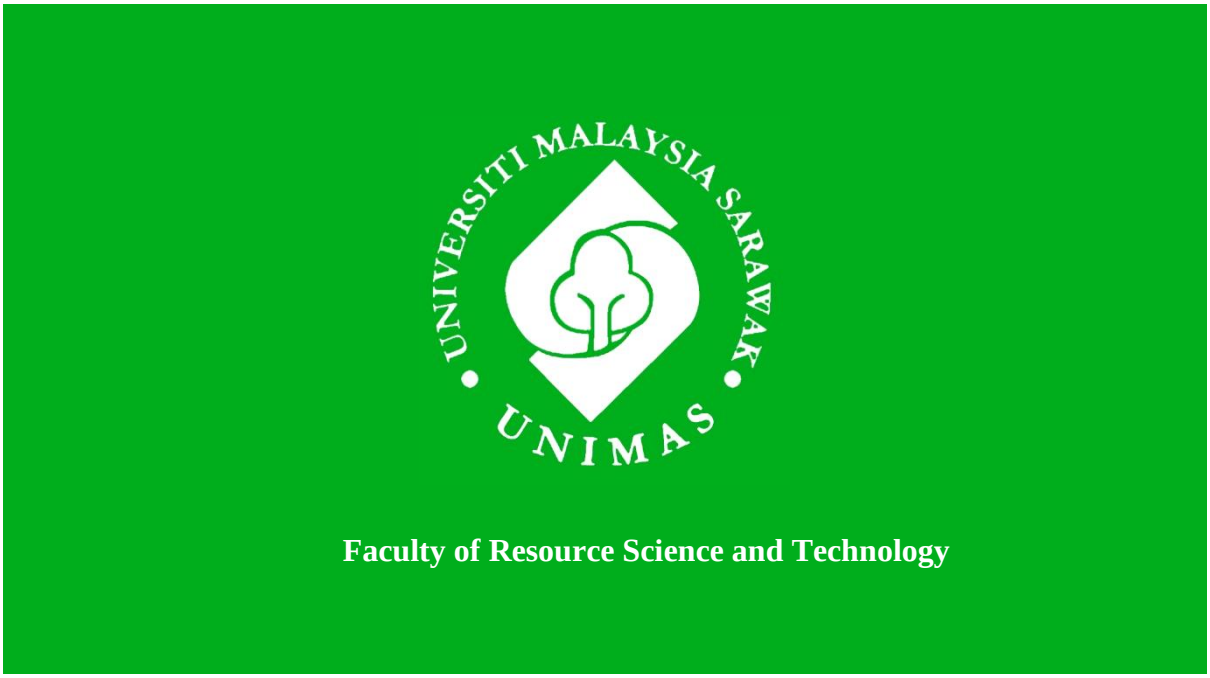

Faculty of Resource Science and Technology

Application of the Real-Time PCR for the Detection and Enumeration of *Vibrio parahaemolyticus* from selected Shrimp Pond

Ikwannuddin Bin Yusuf

26453

Bachelor of Science with Honours
(Biotechnology Resource)
2013

Application of the Real-Time PCR for The Detection and Enumeration of *Vibrio Parahaemolyticus* from selected Shrimp Pond

Ikwannuddin bin Yusuf

26453

A thesis submitted in partial fulfillment of the

Final Year Project (STF 3015)

Supervisor: **Dr Micky Vincent**

Resource Biotechnology

Department of Molecular Biology

Faculty of Resource Science and Technology

Universiti Malaysia Sarawak

30/06/2013

ACKNOWLEDGEMENTS

Thanks to Allah S.W.T, by His will, this project was finally accomplished.

Firstly, I would like to express my honest appreciation and deepest gratefulness to my obliging supervisor, Dr. Micky Vincent for his endless effort, valuable guidance and inspiration which enabled me to successfully complete my study.

I would also like to thank all the postgraduate students in the Microbiology Laboratory, especially Miss Velnetti Linang, for their attention, advices and setting aside time from their busy schedules to help me. Not forgetting Mr. Aziz for his continuous assistance. Without their assistance it would have been impossible to acquire and use the materials and equipment as required. My thankfulness also extent out to all my lab mates for being such delightful, understanding and supportive friends.

My truthful gratitude also goes to my loving family, especially my parents for their unfailing love, consistent prayers and endless support. Last but not least, thank you to all my lecturers, course mates and everyone that has contributed either directly or indirectly towards the completion of this project.

Declaration

I hereby declare that this thesis entitled "Application of the Real-Time PCR for The Detection and Enumeration of *Vibrio parahaemolyticus* from selected Shrimp Pond" is the result of my own research work and effort. It has not been submitted anywhere for any award. Where other sources of information that have been used, they have been acknowledged.

Signature : _____.

Name : Ikwannuddin Bin Yusuf

Date :

TABLE OF CONTENTS

LIST OF ABBREVIATIONS

%	Percentage
^{o}C	Degree celcius
V. parahaemolyticus	*Vibrio parahaemolyticus*
tl	Thermolabile hemolysin
RT-PCR	Real-time Polymerase Chain Reaction
Spp	Species
FOA	Food and Agriculture Organization
PL	Post larvae
DNA	Deoxyribonucleic acid
µl	Microliter
nm wavelength	Nanometer Wavelength
rpm	Revolutions per minute
CFU	Colony Forming Unit
TCBS	Thiosulphate Citrate Bile Salt Sucrose
NaCl	Sodium Chloride
ATCC	American type culture collection
LB	Luria Bertani

LIST OF FIGURES

LIST OF TABLES

Application of the Real-Time PCR for The Detection and Enumeration of *Vibrio parahaemolyticus* from Selected Shrimp Pond

Ikwannuddin bin Yusuf

Resource Biotechnology Programme

Faculty of Resource Science and Technology

Universiti Malaysia Sarawak

ABSTRACT

Vibrio parahaemolyticus is a rod shaped, gram negative bacterium. It is an obligate halophile usually found in the marine environment. The bacterium has been the major cause of food poisoning and gastroenteritis in many countries like the US and Japan. In this study, water samples was collected from selected shrimp ponds (Asia Aquaculture Sdn. Bhd) in Bako, Sarawak and analyzed with real-time PCR using SYBR green chemistry to detect and enumerate the *V. parahaemolyticus* population in the water samples. We had performed these analyses to investigate the dynamic of *V. parahaemolyticus* throughout the shrimp culture period. At the end of the project, the dynamic of *V. parahaemolyticus* shows a decreasing population for pond 1 and increasing population for pond 14. The results show that R^2 value for pond 1 was 0.3073 and pond 14 was 0.3735. It is considered that real-time PCR assay allows more rapid detection and identification of *V. parahaemolyticus* which is faster than current conventional assay.

Key words: *Vibrio parahaemolyticus*, real-time polymerase chain reaction (RT-PCR), SYBR Green.

ABSTRAK

Vibrio parahaemolyticus adalah berbentuk rod, bakteria negatif gram. Ia adalah sejenis halophile yang biasanya ditemui dalam persekitaran marin. Bakteria telah menjadi punca utama keracunan makanan dan gastroenteritis di banyak negara seperti Amerika Syarikat dan Jepun. Dalam kajian ini, sampel air telah diambil daripada kolam udang yang dipilih (Asia Aquaculture Sdn. Bhd) di Bako, Sarawak dan dianalisis dengan real-time PCR menggunakan SYBR kimia hijau untuk mengesan dan menghitung kedinamikan *V. parahaemolyticus* dalam sampel air. Kami telah melakukan analisis ini untuk menyiasat populasi dinamik *V. parahaemolyticus* sepanjang tempoh kitaran hidup udang. Pada akhir projek ini, pola dinamik *V. parahaemolyticus* menunjukkan penurunan populasi untuk kolam 1 dan peningkatan populasi untuk kolam 14. Keputusan menunjukkan bahawa nilai R^2 untuk kolam 1 adalah 0.3073 dan kolam 14 adalah 0.3735. Ia dianggap bahawa asai real-time tindak balas rantain polimerase boleh mengesan & mengenalpasti *V. parahaemolyticus* yang lebih cepat daripada assay konvensional semasa.

Key words: *Vibrio parahaemolyticus*, asai real-time tindakbalas rantaian, SYBR kimia hijau.

CHAPTER 1

INTRODUCTION

1.1 Introduction

Shrimp production has been developing tremendously in the past few years in Malaysia. In the 1970s until the 1980s, the development of shrimp pond started with only 40 farms in Selangor and a model hatchery in Melaka. Also in this period of time, the worldwide shrimp industry have been dependent heavily on Post-Larvae (PL) from wild sources and, only in 1990's that the shrimp industry has been transformed into a large scale industry by using domesticated stocks of PL (Flegel *et al*., 2008). This is due to the low production and the vulnerability of wild PL to disease. Although the facilities have been developing in the past years, the numbers of shrimp production have been modest and variable. Due the fact that the demands of seafood are increasing, shrimp farming has become a potential business to be explored and relevant technical and scientific information is required by new individuals who wish to explore the shrimp farming business. Based on a study done by the Food and Agriculture Organization (FAO, 2001), shrimp PL require brackish environment for survival.

There are several challenge faced by the shrimp industry and the outbreak of infectious disease by various microorganisms caused by uncontrolled development of shrimp farming is a major one (Ligthner *et al*., 1992). If no safety measures are taken during the handling of shrimp, then the shrimps will be contaminated with pathogen and disease. This will then affect the health of the consumers and may lead to food borne disease (Bjorklund, 2006).

Food borne diseases have various effects to the person infected as different microbes that infect the food is also various. Even though the effects can be various, many symptoms manifest as

common disturbance in the digestive system, and, the person suffering from food borne disease usually have diarrhea and vomiting episodes (Landau, 2010). One of the disease caused by eating contaminated or raw seafood is gastroenteritis. Most of the *Vibro spp* will cause this disease. There are three *Vibrio spp*, namely *Vibrio cholerae*, *Vibrio parahaemolyticus* and *Vibrio vulnificus* that may lead to the gastroenteritis in human.

V. parahaemolyticus can be found in raw and contaminated seafood. It can cause gastroenteritis with symptoms ranging from self-limiting diarrhea to cholera like-illness. Usually bloody and watery diarrhea is observed less frequently besides abdominal pain, vomiting, fever, headache, nausea and chill (Fratamico *et al*., 2005). *V. parahaemolyticus* has been the cause of increasing food poisoning in countries like the US and Japan. It usually affects people in countries that consume a lot of seafood. In 1997 until 1998, 4 multistate outbreaks have been recorded in the USA because of the consumption of raw and undercook oyster, with 700 individual affected. In addition, according to a clinical data, an outbreak in 2004 that happened in Spain was connected to the seafood harvested from the European water (Lawley *et al*., 2008). The virulence factor of *V. parahaemolyticus* is the thermolabile hemolysine (*tl*) gene. This heat exotoxin is produce by a few other bacteria, and, can cause the lysis of the Red Blood Cell (RBC).

There are many ways to identify and verify the presence of *V. parahaemolyticus*, for example the usage of alkaline peptone water and Thiosulphate Citrate Bile-Salt Sucrose (TCBS) agar. However, many of these procedures can be time consuming and not accurate. Currently, Polymerase Chain Reaction (PCR) has been developed to detect the specific range of target gene in any sample (Singh *et al*., 2002). Real-Time Polymerase Chain Reaction (RT-PCR) technique is applied due the effectiveness of its mechanism to amplify and detect the deoxyribonucleic

acid (DNA) sequence. Blackstone *et al* (2003) reported the ease and feasibility of detecting the presence of pathogenic *V. parahaemolyticus* in oyster.

In this study, detection and enumeration of *V. parahaemolyticus* was performed by using the real-time PCR based on the SYBR green assay. SYBR Green is chosen due to the fact that it is more economical, rapid and sensitive (Dorak, 2007).

Thus, the objectives of this study are to:

1. Determine the presence of *V. parahaemolyticus* from selected shrimp pond in Bako, Sarawak by using real-time PCR.
2. Enumerate *V. parahaemolyticus* and analyze the result and graph obtain by Real-Time PCR.
3. Study the dynamic of *V. parahaemolyticus* growth during the shrimp culture period.

CHAPTER 2

LITERATURE REVIEW

2.1 Penaeus monodon

Penaeus monodon (shrimp) is a marine crustacean that is mainly captured for source of food. It can be found in the Indo-West Pacific Ocean. This crustacean can grow up to 13 inch in size and the female can exceed 200 g in weight (Dore & Frimodt, 1987). The shrimp body consists of two parts which are the carapace and the abdomen. The carapace is the shell over the cephalothoraxes which consist of the head, vital organ and stomach, while the abdomen consists of six segments (Figure 1). *P. monodon* is also known as giant tiger prawn in some countries in the Indo-West Pacific ocean region (Dore & Frimodt, 1987). The habitat *P. monodon* is more likely in the mud and sand bottom environment from shallow to the depth of 110 m (Dore & Frimodt, 1987). It is often caught offshore and inshore and has become the major aquaculture shrimp species in Asia.

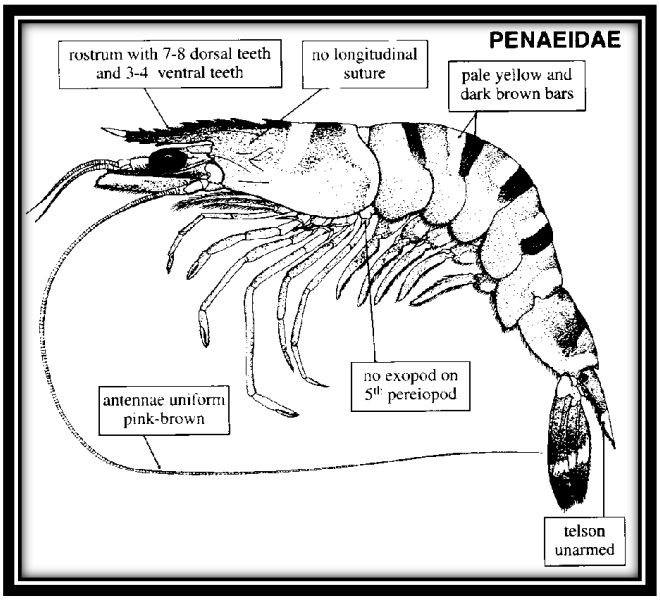

Figure 1: Tiger prawn

Image source: www.fisherieswiki.org

2.2 Vibrio parahaemolyticus

Vibrio parahaemolyticus is a gram negative, motile and a facultative anaerobe (Figure 2) which can survive in a little amount of oxygen (Acha & Szyfres, 2001). It belongs to the family of *Vibrionaceae*. The bacterium is a non-spore forming and an obligate halophyte that usually found in marine organism, often in raw undercook seafood (Lawley *et al*., 2008). Hence, the bacterium has been the leading cause of food borne disease outbreaks in Japan and Korea (Lee *et al*., 2001). It was first identified as the cause of food borne disease in Japan in 1950 (Pruzzo *et al*., 2005) and can cause wound infections and septicemia in susceptible host in minor cases (Morris & Black, 1985).The best media for *V. parahaemolyticus* to develop is usually supplemented with 2% to 3% of NaCl (Acha & Szyfres, 2001). There are two types of strain that has been detected in *V. parahaemolyticus* that are kanagawa positive, which is the cause of food poisoning, and kanagawa negative, which do not cause food poisoning (Lawley et al., 2008). *V. parahaemolyticus* is dormant and inactive during cold season by living in the sea sediment while in warmer season can be found mostly on coastal water, shellfish and fish (Acha & Szyfres, 2001). *V. parahaemolyticus* have the ability to switch to a dormant stage when expose to the inappropriate condition to grow termed as viable but nonculturable (VBNC) (Pruzzo *et al*., 2005).

Figure 2: *Vibrio parahaemolyticus*

Image source: microbewiki.kenyon.edu

2.3 Thermolabile haemolysin (*tl*) gene

Thermolabile can be defined as the change or decomposition of a subject in response to heat while hemolysin is an exotoxin produce by certain bacteria and able to lyses the red blood cell. Haemolysin can act as a lysing agent to the erythrocyte membrane, releasing iron-binding proteins which are haemoglobin, transferring and lactoferrin (Zhang & Austin, 2005). Thermolabile hemolysin (*tl*) is a species specific hemolysin making it a useful target for the detection of total *V. parahaemolyticus* (Yamazaki *et al*., 2008). This gene is present in all *V. parahemolyticus* and not associated with pathogenicity that can cause severe gastroenteritis disease (Liu, 2011).

2.4 SYBR green dye

SYBR green is usually used in molecular biology and biochemistry to obtain a precise result when DNA staining is done. It is much safer than ethidium bromide, less carcinogen and do not bind to DNA in solution (Walker & Rapley, 2008). SYBR green is a fluorogenic minor groove-binding dye that displays little fluorescence when present in solution but produce a strong fluorescence signal when bound to dsDNA and it also functions as nonseqeuence-specific fluorescence adding agent which directly measure DNA amplicon production (Meyer *et al*., 2009). The application of SYBR green is less expensive compared to other chemical and it does not require the synthesis of a target specific probe. Furthermore, it can be used with any pair of primer to measure any gene (Walker & Rapley, 2008).

2.5 *Vibrio parahaemolyticus* selective media

The common selective media used for the isolation of *V. parahaemolyticus* are Thiosulphate Citrate Bile Sucrose (TCBS) agar and CHROMagar. TCBS agar inhibit the growth of gram positive bacteria by incorporation of ox gal and the successful *V. parahaemolyticus* culture will form 2-3 mm green colonies (Riemann & Cliver, 2006). In addition, the *V. parahaemolyticus* colonies are opaque, have raised center and translucent periphery (Parija, 2009). TCBS consist of animal and plant protein, mixture of bile salt, 1% of sodium chloride, sodium thiosulphate, ferric citrate, sucrose and yeast extract. Another selective medium that is often used for *V. parahaemolyticus* monitoring is CHROMagar. The medium contain enzymatic substrates that are linked to chromogenic compounds and the mechanism allow specific enzyme to cleave the substrate and produce colour, usually mauve for *V. parahaemolyticus* and turquoise for *Vibrio vulnificus* (Isenberg, 1998). The colour produce will confirm what type of bacteria present in the culture media.

2.6 Real-time Polymerase Chain Reaction (RT-PCR)

Real-Time Polymerase Chain Reaction (RT-PCR) is a technique to observe the progress of each cycle in the PCR process. RT-PCR uses a fluorescence signal to detect and bind to the double stranded DNA PCR product (Dorak, 2007). The real-time PCR have a wide linear dynamic range, high in sensitivity and very quantitative. The conventional PCR-based methods are sensitive and specific but it require post-PCR detection procedures like the gel electrophoresis which require more time and labor (Blackstone *et al*., 2003).

There are two type of real-time PCR chemistry which is Taqman and SYBR green. The Taqman probe of real-time PCR application act as a hydrolysis probe which increase the specificity of real-time PCR, while the SYBR green probe bind to the double stranded DNA and emit light. There are several studies that report RT-PCR is a rapid and sensitive procedure for the detection of *V. parahaemolyticus* strain in a small amount (Kim *et al*., 2008). The standard curve and the comparative threshold method are the strategies that usually practice to quantify the result acquired by RT-PCR (Raymackers *et al*, 2009). The machine shown in Figure 3 is used in this study.

Figure 3: Real-Time PCR Machine (Located at Forest Genomic Lab, Level 2, FRST, UNIMAS)

CHAPTER 3

MATERIALS AND METHODS

3.1 MATERIALS

The materials that were used in this study are listed below:

1. Shrimp pond water samples
2. PCR thermocycler (Mastercycle personel, Eppendorf ®, USA)
3. Real-time PCR thermocycler (Rotor gene 6000, Corbett Research, Australia)
4. Powersoil kit (MO BIO Laboratories Inc, US)
5. Hemocytometer
6. Water bath
7. Distilled water
8. Ice
9. Stomacher bags
10. RT gene SYBR green PCR mastermix (Qiagen, USA)
11. MV2B primer:
    i. MV2B TLF : 5'-GTT GCA CTC GGT GAC AGC TTG-3'
    ii. MV2B TLR : 5'-AGT TTT GCG TAG GTT AAG TAC-3'

3.2 METHODS

3.2.1 Sample collection

Pond water samples were collected from Pond 1 and Pond 14 in Farm 1 provided by the Asian Aquaculture Sdn. Bhd in Bako, Sarawak (Figure 3 & 4). Sampling were done every week every week for three months until the shrimp was ready to be harvested starting from November 2012 until February 2013. Every week, three tubes of water samples were taken for each ponds by using 15 ml falcon tube and each samples were separated to avoid cross contamination. The samples were transported back to the lab within two hours of collection. During collection, the samples were placed in sterile bags and were placed in ice prior to transportation.

Figure 4: Map showing sampling sites

Figure 5: Shrimp pond at Asian Aquaqulture Sdn Bhd

3.2.2 Standard curve construction

A standard curve was constructed in order to measure *V. parahaemolyticus* in the tested samples intended for the mathematical conversion of Cycle Treshold (Ct) values into bacterial cell figure. The Ct value is the cycle number that formed when a fixed amount of DNA is made based on a series of samples which usually have a known input (Ramakers *et al*., 2004).

3.2.2.1 Preparation of serial dilution

Sterile saline solution was prepared and 9 ml of the solution was poured aseptically into 7 tubes each. Then, 1 ml of the overnight culture of *V. parahaemolyticus* was added to the first tube and mixed thoroughly using vortex. Another 1 ml was taken from the first tube and added to the second tube and mixed well. From the second tube, another 1 ml was taken and introduced into the third tube and mixed well. This procedure was repeated until the seventh tube.